On the Synthesis of Some Protein Salts

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY, IN THE FACULTY OF PURE SCIENCE OF COLUMBIA UNIVERSITY

BY

WALTER HOLLIS EDDY, B.Sc., M.A.

NEW YORK CITY

1909

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DEDICATION.

Inscribed, as a token of my appreciation of interest shown and help given me, to my grandmother,

ELIZA DAY HILDRETH.

ACKNOWLEDGMENT.

The nature and general scope of the work embodied in this dissertation were suggested to me in the Spring of 1907 by Professor William J. Gies, and the investigation has been carried out under his direction.

The author wishes to take this opportunity to thank him for his interest in the work, his advice and counsel at all stages of the work and the many personal sacrifices of his time and convenience to the furthering of its progress. W. H. E.

Laboratory of Biological Chemistry,
College of Physicians and Surgeons,
Columbia University, May, 1909.

INTRODUCTORY.

"We know practically nothing about the ways in which proteins are held in living protoplasmic structures. The molecules of the protein compounds are relatively so large that as yet our physicochemical methods of measurement are inadequate to give us correct ideas of molecular size and intramolecular structure although we know considerable about the lifeless and, in some respects, comparatively meaningless fragments into which protein molecules may be broken. Our ignorance of the fundamental biochemic relations of proteins to the associated constituents of protoplasm appears to be largely due to our vague knowledge of the chemical statics and dynamics of true cellular proteins, a fact which serves as a constant stimulus to protein investigation."

"Among the many protein substances the nucleo-proteins and the gluco-proteins are very important tissue constituents. The essential part of each natural type of these proteins .... appears to be a colloidal organic acid. The form in which the acid radicals occur in cells and tissues has not been definitely made out although it is natural to presume that the acid radicals are united directly to one or more distinctly basic radicals in each case and that variations in the characters of such compounds are associated with the physiologic as well as pathologic variations of cellular composition and tissue function."

"I have endeavored to get to the bottom of this complicated matter, by making definite salts of these proteins with the aid of methods that could be relied upon to leave the acid radicals unchanged. In this effort I have been encouraged by the hope that, by increasing our knowledge of artificial products, analogies with natural compounds would not only be more easily recognized but also the characters and functions of the compounds in living protoplasm would be more thoroughly

understood. Thus, at present it is impossible to recognize satisfactorily the form or forms in which mucoid is contained in the connective tissues under either physiologic or pathologic conditions, nor are we able to explain for example, the possible relations of spermatic protamin (basic) and ovular mucoid (acid) so conspicuous in embryonic tissue. I believe that a study of artificial products of such types as those referred to, will make it possible successfully to elucidate numerous problems awaiting solution, many of which my coworkers and myself have under investigation."[1]

The foregoing quotations present clearly the object of the following research. It was with a view to contributing to the purpose expressed in the above statements that the investigations herein described were begun in 1907, under the direction and counsel of Professor Gies. At that time Gies and collaborators[2] had established the following facts in regard to tendon mucoid and its compounds.

ESTABLISHED FACTS CONCERNING TENDON-MUCOID COMPOUNDS.

1. Tendon-mucoid prepared by the method of Cutter and Gies[3] is, when dried without dehydration in alcohol and ether, a flaky, transparent material of the color of fish glue. On addition of water these flakes take up the water, swell and become soft and creamy white. They dissolve only slightly in water. Particles in this condition give a distinctly acid reaction to litmus, when for example, they are brought in contact with litmus paper. The water used in soaking them gives no reaction.

[1] "Mucoid Salts;" Wm. J. Gies; Coll. Phys. and Surgeons, N. Y. City. (Read in the section on Pathology and Physiology of the American Medical Association at the Fifty-seventh Annual Session, June, 1906.)

[2] Gies and collaborators: Biochemical researches, 1903, i.

[3] Cutter and Gies: Amer. Journ. Physiology, 1902, pp. 155-6.

2. When mucoid is ground in a mortar with an excess of $Ca(OH)_2$ a viscid solution is formed which, on filtering, gives a clear opalescent filtrate. This filtrate is an alkaline solution of calcium-mucoid. The reaction involved may be roughly indicated as follows:

$$\underset{\text{(Acid)}}{\text{Mucoid}} + \underset{\text{(Base)}}{Ca(OH)_2} = \underset{\text{(Salt)}}{\text{Ca-Mucoid}} + \text{Water} + \text{Excess Base.}$$

The excess of base may be removed by dialysis.

(*a*) *Calcium-mucoid* is soluble in water and can be repeatedly precipitated with 95 per cent. alcohol and redissolved with water.

(*b*) The water solution froths on shaking and gives a strong biuret reaction.

(*c*) The water solution gives a flocculent precipitate of mucoid upon addition of a few drops of 0.2 per cent. HCl.

(*d*) It is decomposed on boiling with concentrated HCl. The solution responds positively to the Molisch test and gives a reduction when treated with Fehling's solution, showing that the soluble protein is a gluco-protein.

(*e*) The purified product differs markedly in appearance from the original tendon mucoid.

3. Sodium, potassium and ammonium salts of mucoid (all soluble in water) may be prepared by the same method as that given for calcium mucoid. These, together with calcium mucoid, are light, snow-white powders. "They all dissolve readily in water and are dissociable products."[1] They exert marked osmotic pressure.

"The concentrated solutions resemble mucoids. The aqueous solutions are neutral to litmus and acid to phenolphtalein. Ammonium compounds have been prepared which are acid to litmus also. The calcium salt yields about 12 per cent. of ash, whereas the corresponding mucoid is practically ash free. It is very probable that the mucins in secretions occur

[1] Mucoid Salts; Gies: loc. cit.

in the form of such salts as Müller suggested. Yeast nucleoproteins yield similar products."[1]

PREPARATION OF ALKALOIDAL-PROTEIN COMPOUNDS.

The synthetic results with calcium-hydroxide and stronger bases suggested at once the possibility of bringing about the production of compounds with weak bases, such as alkaloids, protamins, histons, etc. The alkaloids were selected for the preliminary experiments and the work of the present paper begins with these experiments. An abstract[2] of the results obtained was first published in 1907 but the details of the method used is here reported for the first time.

PREPARATION AND PROPERTIES OF MORPHIN-MUCOID.

In these experiments chemically pure, pulverized morphin was used and each sample was subjected to tests to make sure of the absence of free alkali. The tendon mucoid was prepared by the method of Cutter and Gies but dehydration with ether and alcohol was omitted. The dry flakes were washed in distilled water until they became soft; and the washings were frequently renewed and tested to make sure of the entire absence of free acid.

PREPARATION.—Several preparations of morphin-mucoid were made and various methods of securing combination of the two substances, purifying the product and testing the compound were tried. The following summary represents the method finally selected as the most satisfactory of those tested:

1. The acid-free mucoid (previously softened by soaking in water) was thoroughly macerated in a mortar with an excess of morphin. A very little water was used to aid in

[1] Mucoid Salts; Gies: loc. cit., 1906.

[2] Eddy and Gies: Proc. Soc. f. Exp. Biol. and Med., 1907, 4, p. 145.

making an intimate mixture. (Evidence of chemical activity was seen in the peculiarly viscid, smeary character of the mixture. Mucoid and water give a thick milky mixture but it is not viscid or smeary.)

2. A little more water was added to dissolve the compound completely.[1] The mixture was filtered through a wet, platted, hardened filter paper. The first portion coming through was apt to be turbid but when this was put back upon the paper a clear opalescent filtrate was obtained which was neutral to litmus.

3. The filtrate was then put into a parchment bag, which was suspended in a jar of distilled water and the water renewed several times at intervals of 6–24 hours until it gave no morphin test, even when concentrated by evaporation at 40° C.

4. The solution was then removed from the bag and evaporated to dryness on a water bath at 40° C. The dried flaky product was then ground in a mortar and extracted three times with large excesses of ether for the removal of all possible traces of free morphin which might have been carried along mechanically in the filtration process and had not been eliminated completely by dialysis.

5. The product was next dissolved in water and this solution evaporated at 40° C. to a very small bulk. The filtrate was entirely neutral to litmus at this point.

6. The concentrated filtrate was then poured into a mixture of 1/3 ether and 2/3 alcohol and a copious precipitate was formed. This precipitate was *gelatinous* and dissolved easily in water. After dissolving and reprecipitating three times, the final solution was used to test for evidences of a compound.

Evidences of the Production of a Compound. (*Morphin-mucoid.*)—1. The product was soluble in water and precipi-

[1] It is probable that the compound is hydrolytically dissociable.

table by the alcohol-ether mixture, facts proving definitely that it was neither free morphin nor free mucoid, nor a mechanical mixture of the two.

2. The water solution frothed strongly on shaking and gave a strong biuret test, demonstrating the protein character of the solute.

3. The water solution was precipitable by 0.2 per cent. HCl, giving a white flocculent precipitate with that reagent and thus proving the prior presence of a mucoid salt.

4. The alcohol-ether precipitate was gelatinous and not at all like a free mucoid precipitate in ether-alcohol.

The attempts to establish the presence of morphin in the compound were less successful. In fact, the negative character of these tests led finally to the abandonment of morphin for experimentation and the substitution of strychnin as a base.

After considerable experimentation the iodic test was chosen as the most delicate of the preliminary morphin tests and was used in all the attempts to determine the presence or absence of morphin.

This test was applied as follows: 1 cc. of the solution to be tested was added to an equal amount of dilute sulfuric acid. To this was added a few cc. of iodic acid solution and finally a little chloroform. After vigorous shaking, the presence or absence of a violet coloration in the chloroform served to indicate the presence or absence of morphin.

As noted above the results in the main were negative. In the early stages of the preparation positive tests were obtained but after washing with ether and reprecipitating several times positive tests did not result.

Further experimentation with known amounts of morphin sulfate and water showed that while the iodic test was delicate it was possible to have present an appreciable amount of morphin and still get a negative test. This was further

confirmed by concentrating the first solutions which failed to give the test and so getting it.

The conclusion was then, that a compound was formed but that the small amount of morphin present and the lack of delicacy of the test accounted for the failure to discover it in the final compound.

Some Difficulties that Were Met in the Preparation.—1. The compound first made filtered very slowly. It was found that if a large excess of morphin was used the compound formed more readily; there was relatively less insoluble mucoid residue and filtration was facilitated.

2. The precipitation of the purified product with alcohol became more difficult with the increasing purity of the product. Ammonium sulfate precipitated the product but long dialysis was required to remove the salt. Ether alone was not effective as a precipitant. One volume of ether to two volumes of alcohol was found most satisfactory for this process.

3. The failure to detect morphin in the purified products was made the subject of a special investigation. Experiments demonstrated that this result was not due to the quality of the Iodic acid used or to interference from the presence of mucoid. Evidence seemed to point to the gradual dissociation of some of the morphin by the purification methods (hydrolytic dissociation), to the very small amount of morphin in the final product, and to the lack of delicacy of Iodic acid as a test, as the chief reasons for the negative outcome.

PREPARATION AND PROPERTIES OF STRYCHNIN-MUCOID.

PREPARATION.—Strychnin was selected for this preparation primarily because of the extreme delicacy of the dichromate test and the opportunity this test afforded, therefore, for detection of the strychnin base in any compound that might result. The same care was taken to make sure of the purity of the alka-

loid and mucoid as in the preparation of morphin mucoid. Several preliminary preparations were made. The method described below represents the optimum process and also the process followed in making the best product.

1. The mucoid was macerated in a mortar with a large excess of strychnin and just enough water to insure a thorough mixture. The mixture gave the same indication of chemical activity as in the case of the morphin compound, becoming viscid and smeary with change to a grayish color.

2. A little water was then added to dissolve the compound completely and the mixture filtered on a wet, platted filter. The filtrate came through clear.

3. The filtrate was next put into a parchment bag and dialyzed with distilled water until the dialyzate no longer gave a strychnin test.

4. The contents of the bag were then evaporated to dryness on a water bath at 40° C.

5. The dry residue obtained at the conclusion of the evaporation process was then ground in a mortar and the resulting powder washed thoroughly with ether. Washing was made as follows: The powder was put in a bottle with a large amount of ether and shaken from time to time. The ether was filtered off and tested for strychnin. This was repeated until the ether gave no strychnin test. Over two gallons of ether were used for this purpose in final preparation of a fairly large quantity of the compound.

6. The product was next dissolved in distilled water (solution took place readily) and filtered. It was then precipitated with alcohol-ether mixture, giving a gelatinous precipitate. This process was repeated several times and a portion of the water solution tested each time with 0.2 per cent. HCl. It always gave a flocculent precipitate and was neutral to litmus.

7. The water solution of the precipitate was then evaporated to dryness on a water bath at 40° C. and the dry flakes ground to a powder in a mortar.

8. The ground product was then extracted with chloroform. An interesting result was obtained at this point. It took twenty-six extractions with moderate excesses of chloroform to free the compound from admixed strychnin and to secure a strychnin-free washing. In view of the fact that strychnin is practically insoluble in water and is soluble in ether and chloroform this result seemed difficult to explain except on the theory of dissociation by the action of the chloroform.

9. The final product was then tested to establish the presence of strychnin and mucoid.

Evidences of a Compound. (*Strychnin-mucoid.*)—*I General Tests to Establish Its Protein Character.*—1. The water solution gave a strong biuret test.

2. The water solution was precipitated by saturation with Ammonium or Magnesium sulfate.

3. The water solution gave a flocculent precipitate with 0.2 per cent. HCl and with 4 per cent. Acetic acid.

4. The water solution frothed strongly on shaking and was neutral to litmus.

5. The dried product (dried at 40° C.) formed flakes of a transparent yellow character and of a lighter shade than mucoid.

II. Tests to Determine the Presence of Strychnin.—1. The water solution was intensely bitter to taste.

2. The product gave a distinct violet coloration when treated with concentrated H_2SO_4 and a dichromate crystal.

III. Physiological Strychnin Tests.—Four tests were made upon animals, two on frogs and two on dogs.

A water solution was made of the product and diluted to contain 0.575 mg. per cc. The lethal dose of strychnin sul-

fate being about 2.5 mg. per kilo of weight for a frog and 7.6 mg. per kilo of weight for a dog, amounts of the water solution were taken to contain the equivalent amounts of the strychnin mucoid. The solution was given in each case by hypodermic injection.

In the four cases tried the first injection failed to produce any effect. In the case of the first frog an effect followed a second injection of equal amount but it required three injections to produce opisthotonus. Recovery was complete. In the second frog two injections of an amount equal to the *lethal* dose of strychnin produced opisthotonus but the animal recovered. In the first dog it required double the amount of the *lethal* dose of strychnin to produce hyperesthesia and tetanus and the second dog duplicated the results with the first one. In all these tests the effects came on gradually. In all the cases the effects wore off in from three to five hours and the animals completely recovered.

These tests seemed to indicate that the strychnin was liberated gradually from the compound injected into these animals. They also proved conclusively that the compound contained strychnin. These results have also an interesting bearing upon the attempts of Salant,[1] Brown,[2] and others to prove the power of strychnin to combine with tissue elements when mixed with the contents of the gastro-intestinal canal. No attempt was made to investigate this question at this time but the results at least demonstrate that strychnin can combine with a tissue element such as mucoid.

Having established the possibility of making alkaloid-mucoid compounds, attempts to produce alkaloidal compounds with *other proteins* were next made.

[1] Salant, *Amer. Med.*, 1902, **4**, p. 293; *Ibid.*, 1903, **5**, p. 1027.
[2] Brown, *Journ. Bio. Chem.*, 1906, **2**, p. 149.

ALKALOIDAL COMPOUNDS OF OTHER PROTEINS.

Morphin-nucleoprotein. — Two attempts were made to produce a compound of morphin with the nucleoprotein from yeast cells. Neither was entirely successful in establishing the morphin character of the product but both were carried far enough to indicate the production of a water soluble protein of different character from the original nucleoprotein. The methods of preparation follow:

1. Fresh nucleoprotein was made from yeast by first grinding ordinary Fleischman compressed yeast with sand and water to macerate the cells. The mixture was then washed into a bottle, extracted with 0.3 per cent. KOH and the extract filtered and precipitated with 0.2 per cent. HCl. The precipitated nucleoprotein was redissolved and reprecipitated several times to purify it and finally it was washed free from acid.

2. The nucleoprotein prepared by the foregoing process was then macerated in a mortar with an excess of pure morphin and a little water. The mixture was then diluted with more water and filtered. The filtrate was next precipitated with alcohol and the precipitate filtered off and redissolved in water. The filtrate at this point gave good biuret and acid precipitation tests but failed to give a morphin test.

3. A second preparation resulted like the first. Ammonium sulfate was used to precipitate it instead of alcohol.

See the concluding remarks regarding the morphin-mucoid compound, page 12.

Morphin-caseinogen, etc. — Similar preparations were made of morphin-caseinogen, strychnin- and calcium-caseinogen. They were not carried through the rigid purification tests applied to the strychnin-mucoid but far enough to establish satisfactory water-soluble, biuret, and acid-precipitation tests.

The strychnin and morphin tests were faintly positive in the aqueous solutions of the first alcohol precipitates but nothing further was done with these products.

COMPOUNDS OF TENDON-MUCOID WITH OTHER BASES.

Attempts were made to produce compounds of conin, piperidin, and anilin with tendon-mucoid. The method employed was the same as that with strychnin (p. 15). Purification was less extensive but sufficient to warrant definite conclusions as to compound production.

Conin-mucoid.—Combination with conin was rapid, the entire amount of mucoid going into solution and the mixture filtering rapidly. The product was purified by dialysis and precipitated with alcohol. The water solution gave biuret and acid-precipitation tests but the final product failed to give a conin test with KHgI or phospho-tungstic acid.

Piperidin-mucoid was the most successful of the three. The purified product gave not only the usual protein tests but also a piperidin test with platinic chloride.

3. Anilin was the least successful of the three. Much of the product was lost in handling it and preparation was not repeated. No anilin was demonstrated in the final product.

SUMMARY OF CONCLUSIONS RELATING TO THE ALKALOIDAL-MUCOID COMPOUNDS.

1. Strychnin-mucoid was prepared. The product possessed both protein and strychnin characteristics even after extremely rigorous methods of purification were applied to it.

2. The continuous loss of strychnin from strychnin-mucoid in dialysis, by ether extraction, and by washing with chloroform seems explicable only on the basis of dissociation.

3. The results with the other acid-proteins, caseinogen and yeast nucleoprotein, indicate that they are capable of combining with a base in the same manner as mucoid.

4. Conin, piperidin, morphin, and anilin are capable of combining with mucoid to produce water soluble and acid-precipitable compounds. The amount of combined base in these salts seems to be very small.

5. Strychnin is especially suitable for observations in this connection because of the delicacy of the dichromate test for its detection.

PREPARATION AND PROPERTIES OF OVOMUCIN.

In order to obtain some of the gluco protein of a typical cell it was decided at this point to make a preparation of ovomucin as found in the hen's egg. Ovomucin, described by Hammarsten as the insoluble portion of ovoglobulin and by Osborne and Campbell as ovomucin[1] was shown by Eicholz[2] to be a glucoprotein and hence of the same class of proteins as tendon mucoid.

Following the method indicated by these authors a preparation was made with a view to learning the salt-producing properties of this substance. In connection with the preparation several results were obtained which seemed at variance with those published by other investigators and worthy of mention.

Preparation.—The whites of three dozen hen's eggs were diluted with three volumes of distilled water. This precipitated the product. The precipitate was washed thoroughly with water and these washings continued until they gave no biuret test.

[1] Osborne and Campbell, 23rd An. Report Conn. Agr. Exp. Station, 1900.

[2] Eicholz, Journ. of Physiol., 1898, **23**, p. 163.

In the study of the product special attention was paid to the following points:

1. Solubility in weak alkali.
2. Solubility in water.
3. Solubility in 10 per cent. NaCl.
4. Solubility in weak acids.
5. Evidence of its glucoprotein character.

Properties. *I. Reaction with Weak Alkali.*—Osborne and Campbell and Eicholz used 1 per cent. Na_2CO_3 to dissolve the product. In these experiments half-saturated $Ca(OH)_2$ was used, instead.

1. Maceration of the product in a mortar with a small volume of half saturated calcium hydroxide gave a clear viscid solution, which was too thick to filter until diluted with water. It filtered then very slowly, even on a suction funnel.

2. Neutralization of the filtrate with 0.2 per cent. HCl gave a good precipitate at the neutral point and with a slight excess of the acid. Greater excess converted the solution to a milky condition from which there was no precipitation on standing but after neutralization with NaOH calcium hydroxide solution of the ovomucin gave a strong biuret test and was precipitated. with 4 per cent. acetic acid as well as with 0.2 per cent. HCl.

II. Solubility in Water.—The results obtained agreed in the main with those of Osborne and Campbell and indicated that at least a part of the original product was insoluble in water.

1. The $Ca(OH)_2$ solution when poured into a large volume of water gave a turbid mixture containing floating particles.

2. A small portion of the original product, when shaken in a test tube with water, gave a filtrate which did not respond to the biuret test or acid-precipitation treatment. On the other hand a second portion thoroughly macerated in a mor-

tar with water yielded a filtrate which gave a positive biuret test. This filtrate gave no precipitate with 0.2 per cent. HCl and only a slight cloudiness with 4 per cent. acetic acid.

These results demonstrated that at least a part of the product was *insoluble in* water.

III. Solubility in 10 per cent. NaCl.—1. A portion of the product when shaken in a test tube with 10 per cent. NaCl yielded a filtrate which in turn gave no biuret test. On the other hand maceration in a mortar with 10 per cent. NaCl yielded a filtrate which in turn gave a good biuret test. This filtrate gave a precipitate with acid on standing.

2. The filtrate failed to yield a precipitate when poured into a large volume of water but in view of the action of water upon the calcium hydroxide solution this would be a poor way to recover the product. The 10 per cent. NaCl filtrate was precipitated in alcohol and the precipitate was apparently again soluble in 10 per cent. NaCl. This second solution gave a positive biuret test.

3. The NaCl solution was also precipitated by saturation and half saturation with $(NH_4)_2SO_4$ and on saturation with $MgSO_4$.

IV. Solubility in Weak Acid.—Maceration of the original product in a mortar with 0.2 per cent. HCl gave a solution which could be filtered. The filtrate was precipitated on neutralizing. The precipitate so obtained was soluble in excess of alkali. The acid filtrate also gave a biuret test.

V. Evidence of Glucoprotein Character.—The product was hydrolyzed with HCl. The resultant solution was then neutralized with solid KOH and the neutralized liquid poured into hot Fehling's solution. A red precipitate resulted which did not come down readily owing to the protein present. A portion poured into cold Fehling's solution gave no precipitate on standing but on boiling a red precipitate settled out.

VI. Summary of Properties of Ovomucin.—1. Ovomucin is a glucoprotein mostly insoluble in water.

2. It dissolves somewhat in 10 per cent. NaCl, in 0.2 per cent. HCl and in weak alkali such as $Ca(OH)_2$.

3. Precipitation from acid or alkaline solution takes place at or near the neutral point.

PREPARATION AND PROPERTIES OF OVOMUCOID FROM HEN EGGS.

Neumeister[1] investigated a glucoprotein in hen's eggs which he named "pseudo-peptone." This compound was later studied by Salkowski[2], Ch. Mörner,[3] and Eicholz[4]: and was called by them ovomucoid. Mörner's method was used in the following preparation:

Preparation.—1. The whites of eggs were separated from the yolks, cut with scissors and strained through cheese cloth.

2. The coagulable portion was separated by pouring the strained whites into boiling water slightly acidulated with acetic acid. (Constant stirring was necessary to prevent burning).

3. After the coagulum was strained off the liquor remaining was boiled again and this process repeated until no further coagulum would form. The final yellow liquid was an impure solution of ovomucoid.

Purification.—1. The solution was precipitated with 3 volumes of 95 per cent. alcohol. This alcohol had been distilled over lime and carefully tested to make certain of its neutrality. The precipitate obtained was white.

[1] R. Neumeister, Zeitschr. f Biologie, 1890, **27**, p. 331.

[2] Salkowski, Centralblatt f. d. med. Wissench., 1893, **31**, p. 513 and 706.

[3] C. Mörner, Zeitschr. f. physiol. Chem., 1893, **18**, p. 525.

[4] Eicholz, Journ. of Physiol., 1898, **23**.

2. The alcohol was decanted and the product gotten on a filter paper. It was then scraped off and an attempt made to dissolve it in water. At this point the preparation separated into two portions. One portion was readily soluble in *cold* water. The other portion was insoluble in cold water but readily soluble in *hot* water. In the later preparations much less of the insoluble portion was obtained and there were indications that long standing in the alcohol was responsible for the formation of the insoluble part. The insoluble part was dissolved in hot water and otherwise carried through the treatment applied to the soluble portion. The "insoluble portion" finally became soluble in cold water again and behaved in every way like the other part.

3. Reprecipitation with alcohol and resolution in water were repeated several times. As the product became purer precipitation with alcohol became increasingly difficult. Addition of a few drops of 10 per cent. NaCl solution brought about precipitation in each case. Alcohol-ether mixture failed to do so.

4. The final precipitate dissolved readily in water and was dialyzed in a parchment bag with toluol until the dialyzate gave no chloride test. The solution was acid to litmus at this point.

5. The solution so obtained was evaporated to dryness at 40° C. on a water bath yielding yellow flakes which were converted to a white powder by grinding.

Summary of the Properties of Ovomucoid.—1. The water solution was almost colorless when comparatively dilute but assumed a golden color on concentration.

2. The dry product gave yellow flakes of a transparent character and these were readily soluble in cold water.

3. The ground flakes were convertible into a white powder that was soluble in cold water.

4. The water solution gave a good biuret test, frothed strongly on shaking but did not thread on pouring and was not "thick-flowing."

5. Ovomucoid solution does not yield a precipitate with 0.2 per cent. HCl or 4 per cent. acetic acid.

6. It yields precipitates with phosphotungstic and tannic acids.

7. The pure solution is distinctly acid to litmus.

PREPARATION AND PROPERTIES OF OVOMUCOID FROM SHAD ROE.

In order to determine the character of the glucoprotein in other eggs a preparation was made from shad roe. The method of preparation follows:

1. The eggs were first ground in a mortar with sand to macerate them and the mixture was then boiled in acidulated water to precipitate the coagulable protein. The rest of the method was the same as that already recorded for the preparation of hen ovomucoid. In the final dialysis to eliminate chloride, precipitation occurred.

The final product resembled hen ovomucoid in all of the tests described for the latter product (page 23).

SALTS OF OVOMUCOIDS AND YEAST-NUCLEOPROTEIN.

Potassium-ovomucoid (Hen).—1. Just enough water was added to the powdered ovomucoid to make a smeary mixture. This mixture was acid to litmus. It was then macerated in a mortar with 0.3 per cent. KOH until neutral to litmus, diluted with a little water and filtered. The filtrate was slightly turbid.

2. The filtrate was dialyzed for several days with distilled water in the presence of toluol. It was then precipitated

with alcohol giving a gelatinous precipitate not at all like the original ovomucoid. The precipitate dissolved readily in water.

3. Dissolving in water and pouring into alcohol resulted in a second precipitate similar in properties to the first.

At this point, in view of the results obtained simultaneously in the preparation of strychnin ovomucoid this product was dropped and hence the only evidence of a compound was the changed character of the precipitate and its neutrality.

Strychnin-ovomucoid (Hen).—1. A smeary mixture of the ovomucoid was ground in a mortar with an excess of strychnin. A change in the color of the mixture and in its character was at once evident. A little water was added to dilute the mixture and it was then filtered. The result was a turbid solution at first acid to litmus but becoming neutral on standing.

2. The filtrate was next dialyzed for three days against distilled water and in the presence of toluol. The contents of the bag took up water and as the mixture became more dilute it cleared. The dialyzate at the same time became turbid but gave no protein or strychnin tests. The contents of the bag gave both.

3. The solution in the bag was next precipitated with absolute alcohol giving a mixed cheesy and gelatinous precipitate. The alcohol filtrate obtained by filtering off this precipitate gave an acid reaction and a strychnin test. This latter result suggested dissociation.

4. The precipitate was next dissolved in water (solution complete and rapid) filtered and the filtrate tested. It had become acid in reaction and failed to give a strychnin test. The solution was then reprecipitated with alcohol and the solid product tested. It gave no strychnin test and was acid to litmus.

5. A portion of this precipitate was redissolved in water, evaporated to dryness at 40° C. on a water bath and the flakes powdered in a mortar. With this a new strychnin compound was made as before. The solution was again turbid when filtered and it cleared on dilution with water. The clear solution gave both protein and strychnin tests. It again dissociated on precipitation with alcohol.

These results called attention very forcibly to the peculiar dissociable properties of the compound and it was decided to make new preparations and a careful study of their reactions. At the same time experiments were started to determine the possibility of preparing a compound of strychnin with the nucleoprotein obtained from yeast cells.

Strychnin-nucleoprotein (Yeast).—Fresh nucleo-protein was made by macerating 5 lbs. of compressed yeast with water and toluol (30 cc. of toluol per pound of yeast) in a mortar and extracting with 0.3 per cent. KOH for two days. The KOH extract was then filtered off and precipitated with 4 per cent. acetic acid. This precipitate was redissolved in 0.3 per cent. KOH and reprecipitated with acid several times to purify it. The final product was washed free from acid with distilled water and a portion was used to make the compound. The rest was smeared on a plate to dry at room temperature.

Preparation of the Compound.—1. The moist precipitate was triturated in a mortar with excess of strychnin and a little water. The solution was diluted somewhat with water and filtered. The filtrate was clear and bright yellow. It was not alkaline and gave a precipitate with 0.2 per cent. HCl. It was very bitter to the taste, a fact testifying to the presence of strychnin.

2. The filtrate was put into a parchment bag and dialyzed in the presence of toluol against distilled water for some time. The results of this dialysis follow:

(*a*) At the end of the first day the *dialyzate* was clear, gave no biuret or HCl precipitate, was neutral to litmus and bitter to the taste. The solution in the bag was clear and yellow, gave a strong biuret test, a heavy precipitate with 0.2 per cent. HCl, was acid to litmus and very bitter.

The water in the jar was then renewed and allowed to stand another day.

(*b*) At the end of the second day the *dialyzate* was turbid and when concentrated at 40° C. gave a faint biuret test. It was neutral to litmus and phenolphthalein and gave no precipitate with 0.2 per cent. HCl. It was not bitter and did not give a dichromate test for strychnin. The solution in the bag was turbid, gave a biuret test, was acid to litmus and to phenolphthalein and was precipitated by HCl. It gave a positive but weak strychnin test. Water and toluol were again renewed.

(*c*) At the end of the fourth day material from the solution in the bag had precipitated and water had risen in the bag. The *dialyzate* was turbid and gave a faint biuret test when concentrated. The solution in the bag was filtered and the filtrate and precipitate tested. The filtrate gave no acid precipitation tests and no strychnin tests. It was neutral to litmus.

The precipitate was acid to litmus, gave a biuret but no strychnin test.

The filtrate was dialyzed further but failed to yield a precipitate.

The results of these experiments confirmed our view that dialysis brought about a distinct dissociation of the strychnin and the protein. Part at least of the strychnin and the protein passed out of the bag into the dialyzate, whether as a compound or not no evidence is offered except that tests showed their presence there; and since strychnin is practically insoluble in water this presence is hard to explain except on the basis

of a compound, although it is possible that the associated protein exerted a simple solvent action on the alkaloid.

When an attempt was made to confirm these results with a second product using the portion of the nucleoprotein that had been dried at room temperature no compound would form. This could only be explained on the basis that the drying had changed in some way the combining power of the nucleoprotein and furnished another testimonial to the extreme complexity and variation in character that is possible in these protein compounds. A new freshly prepared nucleoprotein again produced the strychnin compound as before.

Strychnin-ovomucoid (Hen). *Second Preparation.*—The second preparation of strychnin-ovomucoid was made by the method for the first preparation. A turbid filtrate was obtained at first which cleared on dilution like the first. Special care was taken to determine the reactions in this preparation. Examination of the first filtrate after mixture of strychnin and ovomucoid showed that it was actually amphoteric to litmus through acid to phenolphtalein. It gave a good strychnin test. This filtrate was returned to the mortar and ground with more strychnin to see if its alkalinity could be increased. It still remained amphoteric and there was no increase in alkalinity.

Dialysis was next tried under the same conditions as with the strychnin-nucleoprotein. The results follow:

The solution in the bag gradually lost its amphoteric character and finally became acid. It also finally yielded a precipitate and lost its power to give a strychnin test as in the first preparation. The dialyzate gradually acquired a protein content but the frequent renewals made it impossible to determine the disposal of the strychnin. The change from an amphoteric to an acid state and the ultimate precipitation confirmed still further the idea of dissociation.

Strychnin-ovomucoid (Shad).—The solution of shad ovomucoid obtained as described on page 24 was evaporated

at 40° C. to a thick syrupy consistency and then macerated with strychnin in a mortar. Like the hen ovomucoid the shad ovomucoid was distinctly acid to litmus.

2. The mixture was next diluted with water and filtered giving a clear yellow filtrate. This filtrate differed in color from the original solution, was rich in strychnin and acid to phenolphtalein but amphoteric to litmus.

3. The filtrate when rubbed with more strychnin failed to increase in alkalinity and remained amphoteric. Dialysis of this filtrate in water gave the following results:

(*a*) At the end of the first day the *dialyzate* was very turbid and gave a strong biuret but no strychnin test. The main solution had lost its amphoteric power but still gave a good strychnin test. The dialyzate was replaced daily for nearly two weeks.

(*b*) During the first few days after the replacement of the dialyzate referred to above very little apparent change took place in either solution or dialyzate but gradually the turbidity of the dialyzate increased and at the end of 10 days the main solution had lost its power of responding to the strychnin test. Each of the successive dialyzates when concentrated by evaporation failed to give strychnin tests but as they were obtained from day to day the amount in each may have been too small for detection.

The compound therefore differed from the hen ovomucoid compound in taking a longer time to lose its strychnin and in failing to precipitate so quickly in the bag. In other respects its action was identical with the hen ovomucoid.

Conclusions Regarding Strychnin-protein Compounds.— While much remains to be done in determining the character of the compounds described in the foregoing pages and while the question of purification is still open owing to the dissociation effects the experiments to this point seemed to demonstrate clearly:

1. The possibility of making in the laboratory salts of such acid reacting proteins as tendomucoid, ovomucoid, and nucleoprotein.

2. That these salts show characteristics markedly different from the characteristics of the substances which enter into their preparation.

3. That the ease with which these compounds give up all or part of their basic portions under such varying conditions as are supplied by dialysis and the presence of certain precipitants indicates that they are extremely labile and sensitive to environmental conditions. The lability in dialysis is of special significance in view of the fact that the conditions are closely similar to those found in the body cells.

It seems safe to conclude therefore that the combination and dissociation of at least mucoid and nucleoprotein compounds is quite within the realm of possibility as a phase of cell chemistry. The nature of such combinations is evidently not so simple as that of a simple salt but bears a definite relation to it.

PREPARATION AND PROPERTIES OF A PROTEIN BASE. (HISTON.)

The preliminary results with strychnin and the other bases led naturally to the use of a weak *protein* base for the production of similar compounds. Protamin and histon were at once suggested as fulfilling the conditions of basicity. Their occurrence in spermatic material suggests their utilization in the natural synthesis of higher compounds with the acid proteins of eggs such as ovomucoid as such transformations are possibly of great significance in the process of fertilization and in cell chemistry in general.

The recent results of Taylor[1] in which he reports the syn-

[1] Taylor, Journ. of Bio. Chem., 1907, 3, p. 87; Ibid., 1909, 5, p. 381.

thesis of protamin from amino acids with the aid of trypsin gives added interest to the behavior of these simple proteins in the cell.

The availability of thymus as a source of histon and the fact that of the two basic proteins, histon was least basic and would therefore present greater difficulties in the way of compound making, led to its selection for the preliminary work.

In order to obtain the material and to become thoroughly familiar with its properties, histon was prepared from fresh thymus glands of the calf. The methods of extraction selected for preliminary work were those of Huiskamp[1] and Bang.[2] The tabulation on page 32 presents a summary of the various methods used by Lilienfeld,[3] Malengrau,[4] Huiskamp,[1] and Bang.[2] It will be seen by examination of this tabulation that the work of the latter two investigators embodies the methods of the first two and gives opportunity for more complete study of the product. The following account details the properties of histon as determined in these studies.

PRELIMINARY PREPARATIONS OF HISTON.

The preliminary preparations followed the outlines of the Huiskamp and Bang method exactly as tabulated, the preparations being parallel in point of time in order to compare results. Fresh thymus glands were obtained and freed from fat with the knife. They were then minced in a meat chopper and equal amounts used in the first two preparations.

Huiskamp Preparation.—In this preparation the minced glands were extracted for 24 hours with distilled water (600

[1] W. Huiskamp, Zeitschr. f. physiol. Chem., 1901, **32**, p. 145; Ibid., 1901, **34**, p. 32.

[2] Ivar Bang, Hofmeister's Beitrage, 1903, **4**, p. 115.

[3] L. Lilienfeld, Zeitschr. f. physiol. Chem., 1893, **18**, p. 473.

[4] F. Malengrau, La Cellule, 1900, **17**, p. 339; Ibid., 1905, **19**, p. 285.

LILIENFELD METHOD.

Thymus + water
→ Insol. residue
↓
Soluble nucleohiston + 4% acetic acid
→ Sol. material
↓
Ppt. of nucleohiston + 0.8% HCl
→ Insol. leuconuclein
↓
Histon solution + NH_4OH
→ Sol. residue
↓
Histon (purified with alcohol and ether)

MALENGRAU METHOD.

Thymus + water
→ Insol. residue
↓
Soluble nucleoalbumins + 4% acetic acid
→ Sol. residue
↓
Ppt. nucleoalbumins + Na_2CO_3
→ Insol. residue
↓
Soluble nucleoalbumins + 30–40% $(NH_4)_2SO_4$

Ppt. "A" nucalb. + 1% HCl	Sol. "B" nucalb. + 56–72% $(NH_4)_2SO_4$
→ Insol. nuclein	→ Sol. residue
↓	↓
Sol. histon + 45% $(NH_4)_2SO_4$	Ppt. nucleohiston + 1% HCl
→ Sol. resp.	→ Insol. residue
↓	↓
Ppt. histon	Sol. histon + 55% $(NH_4)_2SO_4$
(N. B. Bang claims this is not	→ Sol. nuclein
histon but an acid albumin)	↓
	Ppt. histon

HUISKAMP METHOD.

Thymus + water
→ Insol. residue
↓
Soluble nucleoproteins + $CaCl_2$ to 1%

Ppt. nucleohiston + NH_4OH and H_2O	Sol. nuc. albumin + Ac. (4%)
→ Insol. residue	→ Sol. Res.
↓	↓
Sol. N. histon + $CaCl_2$ (to 1%)	Ppt. nucleoalbumin
→ Sol. residue	(N. B. Contains no histon)
↓	
Ppt. N. histon + 0.8% HCl	
→ Insol. nuclein	
↓	
Sol. histon HCl + dialysis	
→ $CaCl_2$ and HCl, etc.	
↓	
Neutral sol. histon—HCl + NH_4OH	
→ NH_4Cl, etc.	
↓	
Ppt. histon.	

Chemistry of above: Thymus nucleohiston probably exists as Na nucleohistonate soluble in water in the gland.

Na–nucleohistonate + $CaCl_2$ = Ca–nucleohistonate + NaCl.

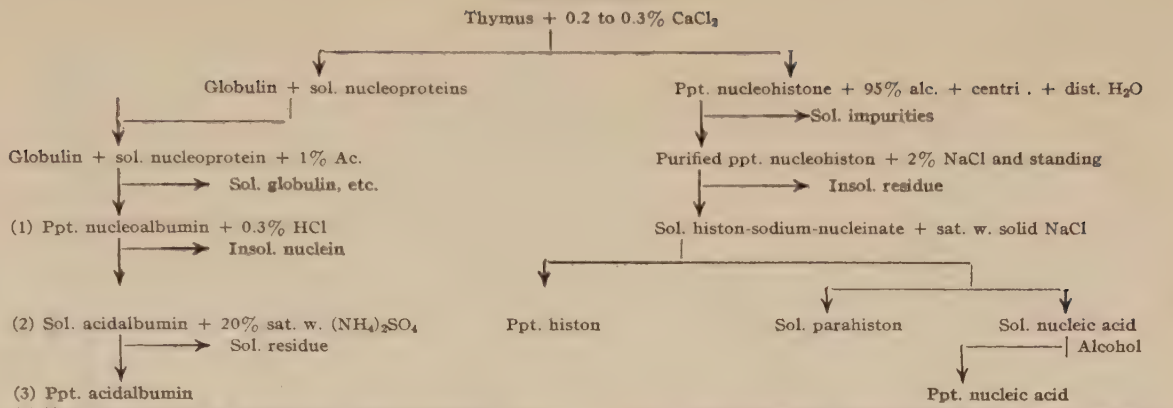
Ca–nucleohistonate + HCl = histon–HCl + $CaCl_2$, etc.

Histon–HCl + NH_4OH = histon + NH_4Cl, etc.

Probable composition of Huiskamp's nucleohiston.

Nucleohiston
↓
Histon | Nuclein
↓
Nucleic acid | albumin

BANG METHOD.


Thymus + 0.2 to 0.3% $CaCl_2$
Globulin + sol. nucleoproteins
Ppt. nucleohistone + 95% alc. + centri . + dist. H_2O
Sol. impurities
Globulin + sol. nucleoprotein + 1% Ac.
Sol. globulin, etc.
Purified ppt. nucleohiston + 2% NaCl and standing
Insol. residue
(1) Ppt. nucleoalbumin + 0.3% HCl
Insol. nuclein
Sol. histon-sodium-nucleinate + sat. w. solid NaCl
(2) Sol. acidalbumin + 20% sat. w. $(NH_4)_2SO_4$
Sol. residue
Ppt. histon
Sol. parahiston
Sol. nucleic acid
Alcohol
Ppt. nucleic acid

(3) Ppt. acidalbumin

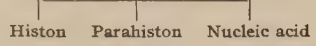
(1) This corresponds to Malengrau's histon-nucleinate and is soluble in 0.1% $CaCl_2$ and 0.9% NaCl.

(2) This is Malengrau's "histon." Bang claims that it is not a histon but an acid albumin. It is precipitable by ammonia.

(3) This is free from P. but contains 16.59% N.

N. B. Bang claims that histon-nucleinate is a better name for the compound from which the histon is split off than nucleohiston, because it does not contain a nuclein.

Bang's histon-nucleinate.



cc. to 200 grams of thymus). This extract was then strained through unbleached cotton cloth and centrifugalized to free it from tissue shreds. The centrifugalized extract was then filtered through wet, platted filter papers. This filtration process was very slow owing to the slimy character of the insoluble residue. The suction funnel was useless on this account. The filtrate finally obtained was turbid.

To this filtrate was added 1 cc. of 10 per cent. $CaCl_2$ solution for every 100 cc. of extract; resulting in a precipitate of nucleohiston and some impurity carried down with it. Centrifugalizing served to separate the precipitate and it was then extracted again with water plus a small amount of ammonia. Solution took place with difficulty and several extractions were necessary to obtain the nucleohiston. Filtration of this solution was very slow.

The filtrate was next reprecipitated with $CaCl_2$ solution as before. The resulting precipitate was again centrifugalized and filtered off and then extracted with 0.8 per cent. HCl to separate the histon as the histon-hydrochloride from the insoluble nuclein. The HCl extract was then dialyzed in a parchment bag with toluol against distilled water until free from chlorides and uncombined acid. When neutral it was precipitated by the addition of ammonia to distinct excess. The product was a white precipitate soluble in water.

Bang Preparation.—In the Bang preparation the thymus was extracted with 0.9 per cent. NaCl solution, which dissolved out the nucleoprotein and the globulin. This extract was then filtered and gave a milky filtrate which was amphoteric in reaction but more basic than acid. The milky extract was next precipitated by adding 3 cc. of 10 per cent. $CaCl_2$ solution to each 100 cc. of extract. The resulting precipitate of nucleoprotein was shaken with 95 per cent. alcohol and centrifugalized. Alcohol was used to lower the specific gravity of the soluble residue. This precipitate was then washed

with distilled water and allowed to stand in the water for some time in the presence of toluol. At the end of that time it was filtered off and rubbed up with 2 per cent. NaCl in a mortar. The resulting soluble histon-sodium-nucleinate was then filtered off and was of a clear, bluish, fluorescent character. An attempt to precipitate the solution by adding an equal volume of distilled water was only partially successful. The solution was therefore next saturated with solid NaCl and the histon precipitated out. The mixture of this histon with the NaCl complicated this step in the process and rendered long dialysis necessary to free it from NaCl.

These two preliminary preparations were mainly valuable in teaching the methods, for owing to the long time required for some of the steps, putrefaction occurred in each case ultimately, in spite of the presence of toluol, and the final products were therefore rejected as not of sufficient purity to justify their use for experimental purposes.

Following these preliminary preparations several preparations were made in order to further investigate the different steps in the process and to learn how better to avoid the difficulties. In these preparations attention was devoted mainly to the Huiskamp method. Putrefaction was avoided by keeping the preparations in a refrigerator.

In the SECOND PREPARATION various strengths of $CaCl_2$ were tried to precipitate the nucleohiston and 5 cc. of 10 per cent. $CaCl_2$ solution to 100 cc. of extract was found to be the optimum. The final ammonia precipitate in this preparation was washed free from ammonia with 90 per cent. alcohol.

In the THIRD PREPARATION, 95 per cent. alcohol was mixed with the $CaCl_2$ precipitate of nucleohiston to make centrifugalization easier. The final product was washed free from ammonia with alcohol and ether. This treatment was not successful, as the product so obtained failed to dis-

solve in water and long extraction with 0.8 per cent. HCl failed to give an ammonia precipitable substance. No explanation has occurred of this anomalous behavior and it can only be surmised that in some way the alcohol and ether washings must have changed the character of the histon and rendered it insoluble in water.

In the FOURTH PREPARATION the muslin-strained water extract of thymus was precipitated at once with $CaCl_2$ without filtering it. The impure precipitate of nucleohiston was then extracted with ammoniated water and the extract filtered off. This extract was then again precipitated with $CaCl_2$ to purify it and this precipitate filtered off and extracted with 0.8 per cent. HCl. Certain suggestive results followed from the dialysis of this extract.

1. At the end of the first day the dialyzate was acid and gave a biuret test. The dialyzate was replaced with distilled water and the process continued.

2. At the end of the next day the dialyzate was again acid and gave a biuret test. Again the dialyzate was replaced with distilled water and the toluol in the bag renewed.

3. At the end of the next two days the dialyzate was no longer acid to litmus though the paper was waved back and forth in it for several minutes and different depths of the dialyzate were thoroughly tested in this manner. Though free from acid the dialyzate still gave a biuret test and a faint precipitate with ammonia. These results seemed to suggest that the histon was itself at least partially dialyzable.

The solution in the bag at this time was found to be neutral. It was filtered off and the filtrate precipitated with ammonia. This precipitate was filtered off, dissolved in a little water and dialyzed in a second parchment bag to free it from ammonia.

4. On the following day this dialyzate contained histon, giving both the biuret test and the precipitation test with ammonia. A few drops of ammonium chloride were added

to this ammoniated dialyzate to see if the histon would flake out. None came. The dialyzate was then replaced with distilled water, which was renewed once in 48 hours. The first 24-hour dialyzate gave a biuret test but no ammonia precipitate. The biuret response was much weaker than in the previous dialyzate. The solution in the bag at this time was found to be distinctly alkaline to litmus but whether this was due to the histon or the free ammonia was of course an open question.

The 48-hour dialyzate was feebly alkaline but gave no biuret test or ammonia precipitate. The dialyzate was then replaced with distilled water every half hour for four hours, which was then allowed to stand for 20 hours.

5. At the end of this time the final dialyzate showed no alkalinity. The solution in the bag was weakly alkaline. This dialysis was continued with several changes of water for two days more. Toluol and the ice box were used throughout this process to preserve the product. At the end of this time the dialyzate showed no alkalinity and it was practically certain that no free ammonia could be present. The solution in the bag was then filtered off. (A portion of the substance had precipitated in the bag). Some of this filtrate was tested with ammonia. Turbidity resulted but no precipitate. A second portion was then treated with ammonia and a glass rod, dipped in ammonium chloride solution and allowed to drain, was used to stir the ammoniacal solution. A fine flocculent precipitate separated out after a few minute's standing. A third control portion was treated with ammonia at the same time and no ammonium chloride added. It remained colloidal for 2 hours but at the end of that time gradual precipitation commenced to occur.

These results with the final histon solution and the ammonium chloride were especially interesting as bearing upon Bang's contention that ammonia precipitates histon from

solution only in the presence of salts. Cohnheim questions this contention. The above results would seem to indicate that precipitation occurs much more readily in the presence of traces of salt but they also demonstrated that precipitation will result in the absence of these salts if the solution be allowed to stand a sufficiently long time.

The dialytic results were made the subject of special investigation.

A portion of the purified histon solution was used in the preliminary attempts at salt formation. (See page 43). The rest was evaporated to dryness upon a water bath at 40° C. It dried in the form of flakes which were readily soluble in water.

Some of the ammonia-precipitated histon just described was treated on a filter paper with a few drops of concentrated HNO_3. The resulting filtrate was then treated with an equal volume of molybdic solution. No PO_4 was indicated. In view of the fact that histon is precipitated by cold HNO_3, this result was principally important in indicating that the histon obtained was free from impurities of a PO_4-containing nature. This precipitation of histon by cold nitric acid and its solubility in hot acid were Kossel's reasons for originally placing histon among the albumoses.

The FIFTH PREPARATION of histon was made primarily to obtain an appreciable amount of neutral histon hydrochloride for the production of histon compounds. The methods were therefore abridged as much as compatible with the securing of a pure product. The method actually used was practically that of Lilienfeld.[1]

1. Thymus was extracted with cold water in an ice box for 24 hours.

2. The extract was then decanted and precipitated with 4 per cent. acetic acid. (Some was precipitated with $CaCl_2$ but this was later dissolved in ammoniacal water and repre-

cipitated with acetic acid after mixing with the original extract).

3. The precipitate of nucleoprotein, consisting, according to Huiskamp of a mixture of nucleoalbumin and nucleohiston, was washed free from acid and then extracted with 0.8 per cent. HCl for 24 hours in the cold.

4. The HCl extract was then filtered and dialyzed until free from acid. The solution in the bag was a neutral solution of histon-HCl and this solution was kept in a stoppered bottle with toluol. It furnished the material for some of the experiments on the production of histon-protein salts.

In this fifth preparation the question arose as to whether the acetic acid used to precipitate the extract of thymus might not dissolve some histon. The filtrate obtained on precipitating the nucleoprotein was treated with ammonia and gave a faint precipitate but it was so slight in amount as to indicate no appreciable loss of histon through this agency.

FINAL PREPARATIONS OF HISTON.

In order to settle all doubtful points in connection with the preparation of histon, four pairs of thymus glands were obtained and four preparations started. The bulk of the material was used to prepare histon-HCl after the Lilienfeld method. Portions were reserved for preparations according to Malengrau, Huiskamp, and Bang. In the previous preparations no attention was paid to the by-products. In this final preparation all of the by-products were investigated. The results follow:

The Lilienfeld Preparation.—Thymus was extracted with water, the extract filtered off, precipitated with 4 per cent. acetic and the precipitate extracted with 0.8 per cent. HCl. The HCl extract gave a precipitate of histon with ammonia and the bulk of the HCl extract was dialyzed free

from acid in the presence of toluol and used to make new salts. For rapid preparation of histon this method is preferable to the other three. It does not, however, insure absolute purity of the product and to determine this point a portion after dialysis was precipitated with ammonia, and washed free from ammonia and soluble protein with alcohol and ether. The purified product was then found to have become insoluble in water in exactly the same manner as that described on page 36. This denaturizing of the histon may be due to dehydration.

The leuconuclein remaining after the extracton with HCl was washed free from impurity with water and was found to be a protein. It was entirely free from histon.

The Malengrau Preparation.—Following, in general, the outline laid down for this preparation in the table on page 32, the thymus was extracted with water, filtered, and precipitated with 4 per cent. acetic acid. This precipitate was then dissolved in sodium carbonate. The sodium carbonate solution was then treated with ammonium sulfate to 40 per cent. saturation. A small portion precipitated out and was extracted with 1 per cent. HCl. The extract gave no precipitate with ammonia, possibly due to the small amount of material. The presence of histon in Malengrau's "A" nucleoalbumin was not demonstrated. This result made it impossible to settle the point here as to whether the histon precipitate obtained by Malengrau was histon or acid albumin.

The 40 per cent. saturated solution was next filtered off and divided into two portions. One portion was neutralized and allowed to stand 3 days with toluol. No further precipitate appeared. The other portion was acidified and left to stand in the same way. No precipitate occurred in this either.

The two portions were then treated with more ammonium sulphate until a precipitate formed. This was filtered off and

extracted with 1 per cent. HCl. The extract was saturated to 55 per cent. with ammonium sulphate and a precipitate of histon resulted.

The Huiskamp Preparation.—Thymus was extracted with water, the water extract filtered off and precipitated with 5 cc. of 10 per cent. $CaCl_2$ solution to each 100 cc. of extract. The precipitate was filtered off and the filtrate precipitated with 4 per cent. acetic acid. The nucleoalbumin obtained from the filtrate with the acetic acid was then extracted with 0.8 per cent. HCl to determine whether it contained histon. The HCl extract gave no ammonia-precipitable substance, confirming Huiskamp's contention that thymus contains two nucleoproteins, one of which (his nucleoalbumin) contains no histon.

The $CaCl_2$ precipitate of nucleohiston was redissolved in water to which ammonia had been added. Solution took place very slowly rendering this process of purifying the nucleohiston very difficult. The filtrate finally obtained was reprecipitated with $CaCl_2$ and this precipitate washed with water and extracted with 0.8 per cent. HCl giving a clear extract, which in turn yielded a copious precipitate of histon when treated with ammonia. Most of the HCl extract was dialyzed free from acid and added to the stock from the Lilienfeld method. The insoluble nuclein was tested to determine whether it was a nuclein or a nucleic acid. After washing free from all protein with water, it gave a strong Millon reaction, demonstrating that it contained a protein body which was not histon as histon gives only a faint Millon test.

The Bang Preparation.—Thymus was extracted with 0.9 per cent. NaCl. The extract was then filtered off and gave the typical amphoteric reaction. (The water extract of Huiskamp was acid to litmus, not amphoteric). The extract was then precipitated with 2 cc. of 10 per cent. $CaCl_2$ solution to each 100 cc. of the extract. The precipitate of nucleo-

histon was filtered off and the filtrate was treated with 1 per cent. acetic acid to precipitate the nucleoalbumin. This nucleoalbumin was washed free from acid and extracted with 0.4 per cent. HCl. The HCl extract was filtered off and gave an ammonia precipitate. On attempting to dissolve this precipitate in water no solution resulted, confirming Bang's view that this precipitate was an acidalbumin and not a histon.

The $CaCl_2$ precipitate of nucleohiston was washed with water until the washings gave no biuret test and then dissolved in 2 per cent. NaCl solution. The resulting solution was then filtered and saturated with solid NaCl. A histon moiety separated out. This histon was filtered off and the filtrate precipitated with alcohol to recover the nucleic acid. This precipitate Bang contends is not a nuclein but a nucleic acid. The alcohol precipitate was filtered off, and dissolved in NaCl. The resultant solution gave no biuret test. The main bulk of this NaCl solution was again thrown into alcohol and thus precipitated. These results seem to fully confirm Bang's contention that the sodium chloride solution of the nucleohiston is a histon-sodium-nucleinate and that when the histon is split off a nucleic acid, not a nuclein, is left.

The histon separated by the NaCl saturation was then dialyzed free from NaCl and used to establish the properties of histon. Before applying tests to the histon, attempts were made to establish the possibility of forming salts with it. The results follow.

PROTEIN SALTS WITH A HISTON BASE.

On obtaining purified histon attempts were made to bring about its combination with acid proteins. For this purpose tendomucoid and yeast nucleoprotein were used. Proceeding on the lines suggested by the behavior of strychnin, attempts were made to secure a water soluble product. Experiments in this direction demonstrated that if a compound

were formed it was not water soluble. Assuming from the results of these experiments that an insoluble compound was formed, experiments to determine this point were conducted as follows:

Preliminary Experiments. (*Histon-mucoid.*)—1. An extract of histon in 0.8 per cent. HCl was made and dialyzed until free from uncombined acid. The solution obtained was presumably histon-HCl and was entirely neutral to litmus.

2. A solution of acid-free mucoid was made with 0.3 per cent. KOH and this solution dialyzed until it was neutral to litmus. This solution was presumably potassium-mucoid.

(N. B. Toluol was used in both these dialyses as a preservative and in the case of the mucoid care was taken to have as little free KOH as possible.)

3. The histon and mucoid solution were then each filtered giving clear filtrates.

4. The filtered mucoid solution gave a heavy precipitate with 0.2 per cent. HCl, gave no precipitate with ammonia, was neutral to litmus and gave a heavy precipitate with a water solution of histon.

The filtered histon-HCl solution gave a heavy precipitate with ammonia, no precipitate with 0.2 per cent. HCl, was neutral to litmus and gave a heavy precipitate when added to the K-mucoid solution.

5. The K-mucoid solution was next added to the histon-HCl solution and a flocculent precipitate appeared. It was found in this connection that a small amount of the histon-HCl would precipitate a very much larger quantity of K-mucoid. When, therefore, histon HCl was added drop by drop it was possible to avoid excess and to produce a sharp separation between the precipitate and the clear supernatant liquid. Excess of either reagent seemed to bring about solution of the precipitate.

6. The precipitate was then filtered off and washed with water until the washings no longer gave a precipitate with ammonia or 0.2 per cent. HCl. The product thus obtained was then tested as follows to establish its properties.

Preliminary Tests to Determine the Nature of the Product. —1. A portion of the precipitate was ground in a mortar with 0.05 per cent. sodium carbonate solution. The mixture was then filtered, resulting in a colloidal filtrate. This filtrate gave a heavy precipitate with 0.2 per cent. HCl. It gave a less heavy but distinct precipitate with ammonia. The precipitates corresponded approximately to the amounts in which the histon and mucoid appeared to combine.

These results left open the question as to whether the sodium carbonate had brought about a cleavage into histon solution and Na-mucoid or had formed a soluble compound Na-histon-mucoid or finally had dissolved the histon-mucoid.

2. (*a*) A portion of the sodium-carbonate filtrate was treated with 95 per cent. alcohol. It failed to precipitate either at once or on standing.

(*b*) A portion of the filtrate was treated with 95 per cent. alcohol and one drop of 10 per cent. NaCl solution. A precipitate appeared on standing.

(*c*) Alcohol-ether mixture alone failed to precipitate the filtrate but on addition of a drop of NaCl solution, a precipitate settled out.

(*d*) The precipitate obtained in (*b*) and (*c*) was filtered off and an attempt made to dissolve it in water. The filtered mixture gave no HCl or ammonia precipitate seeming to indicate at this point that the sodium carbonate produced a simple solution. The result was not conclusive, however, and further experiments with the carbonate solution were made.

3. (*a*) Two solutions were made by (*aa*) adding 5 cc. of histon-HCl solution to 5 cc. of 95 per cent. alcohol and by

(*bb*) adding 5 cc. of histon-HCl solution to 5 cc. of 95 per cent. alcohol plus 3 drops of 10 per cent. NaCl.

(*b*) Two more solutions were made by (*aa*) adding 2 cc. K-mucoid solution to 3 cc. water and then to 5 cc. 95 per cent. alcohol and by (*bb*) adding 2 cc. K-mucoid solution to 3 cc. water and then to 5 cc. 95 per cent. alcohol plus 3 drops 10 per cent. NaCl.

(*c*) Two solutions were made by adding 5 cc. of 0.05 per cent. Na_2CO_3 solution of H-mucoid to 5 cc. of 95 per cent. alcohol and 5 cc. of 0.05 per cent. Na_2CO_3 solution of histon-mucoid to 5 cc. of 95 per cent. alcohol plus 3 drops of 10 per cent. NaCl solution.

(*d*) Another solution was made by adding 2.5 cc. of histon-HCl solution to 5 cc. of 95 per cent. alcohol plus 3 drops of NaCl solution.

(*e*) Another solution was made, composed of 2.5 cc. K-mucoid solution to 5 cc. of 95 per cent. alcohol plus 3 drops of 10 per cent. NaCl solution.

(*f*) A final solution was made containing 2.5 cc. of 0.05 Na_2CO_3 solution of histon-mucoid plus 2 drops of 10 per cent. NaCl solution and 5 cc. of 95 per cent. alcohol.

Results.—1. The histon-HCl solution gave no precipitate in the alcohol either in the presence or absence of salt. The mixture did not yield a precipitate on standing and no turbidity resulted.

2. The K-mucoid solution gave no precipitate in the alcohol alone but did so in the presence of the salt.

3. The sodium carbonate solution of histon mucoid gave a turbidity in both preparations and in the presence of the salt a precipitate settled out on standing.

4. Two volumes of alcohol failed to precipitate histon-HCl in the presence of salt.

5. K-mucoid gave a heavy precipitate with two volumes of alcohol in the presence of salt.

6. The sodium carbonate solution of histon-mucoid gave a heavy precipitate with two volumes of alcohol in the presence of salt.

Next a mixture was made by pouring the alcohol solution of histon-HCl into the alcohol solution of K-mucoid. A precipitate formed at once indicating that combination took place in the presence of alcohol and that the resulting compound was precipitable in alcohol.

Finally the precipitate from the Na_2CO_3 solution of histon-mucoid was filtered off. This precipitate dissolved in water! Furthermore the water solution gave precipitates with 0.2 per cent. HCl and with ammonia and the amounts of the precipitates obtained from equal volumes of the solution were the same proportionally as those obtained with the Na_2CO_3 solution before treatment with alcohol. These results contrary to those obtained on page 44 seem to indicate that the sodium carbonate must have formed with the histon-mucoid a new compound which unlike histon-mucoid is soluble in water. This compound was presumably Na-histon-mucoid.

Conclusions.

The above experiments and the results obtained seemed to prove definitely that histon and mucoid combined to form a water-insoluble compound. Furthermore this compound is soluble in 0.05 per cent. sodium carbonate solution and this solution is apparently due to the formation of a soluble sodium salt, namely, sodium-histon-mucoid.

Again, the results with alcohol demonstrated that both the purified K-mucoid and the histon-HCl are soluble in 95 per cent. alcohol if no salt is present and that their combination will not only take place in the presence of alcohol but the compound so formed differs in properties from both these components by being itself precipitable in alcohol which contains no salt.

In order to further investigate these important points, the following procedure was carried out:

Further Tests to Determine the Nature of the Product.— 1. A new histon-mucoid compound was made by adding histon-HCl solution to K-mucoid solution. Both solutions were previously dialyzed free from acid and alkali respectively and were neutral to litmus. The precipitate was washed free from histon and mucoid with water, the washings being continued until they gave no ammonia or acid precipitates.

2. One portion of the precipitate was then macerated in a mortar with 0.1 per cent. HCl and a second portion in a mortar with 0.1 per cent. KOH. These mixtures were each filtered off and the filtrates tested in each case with ammonia and 0.2 per cent. HCl.

The acid filtrate gave a precipitate with ammonia but none with 0.2 per cent. HCl. A faint cloudiness resulted with the HCl. The alkali filtrate gave a heavy precipitate with HCl but none with ammonia. This latter result was probably due to the splitting off of the mucoid by the alkali with the formation of soluble K-mucoid, or K-histon-mucoid may have been formed in quantities too small to give a histon precipitate with ammonia.

These results may be tabulated as follows:

1. Histon-HCl + K-mucoid = precipitate of histon-mucoid + excesses.

PPt. washed with distilled water until washings gave

(*a*) No ppt. with NH_4OH = removal of excess of H-HCl.

(*b*) No ppt. with 0.2 per cent. HCl = removal of excess of K-mucoid.

2. Pure histon-mucoid + 0.1 per cent. HCl = histon-HCl + insol. mucoid.

3. Pure histon-mucoid + 0.1 per cent. KOH =

(*a*) K-histon-mucoid or

(*b*) K-mucoid + histon-HCl, or

(*c*) K-mucoid + histon-HCl, or

(*d*) K-mucoid + histon + KCl.

The filtrate gave a precipitate with 0.2 per cent. HCl but not with NH_4OH.

Whatever the results in 3 may be, the steps in 1 and 2 left no doubt of the fact of a histon-mucoid compound.

Preparation of Histon-Nucleoprotein.—1. A new preparation of yeast nucleoprotein was made as described on page 26. This was purified by resolution and reprecipitation, and the final product washed free from acid with water.

2. A portion of this nucleo-protein was then combined with KOH to make a soluble potassium-nucleoprotein compound. This compound was dialyzed free from alkali in the presence of toluol and the final product obtained was neutral to litmus.

3. The solution of K-nucleoprotein obtained in 2 was then precipitated with histon-HCl. Much more of the histon solution was needed to secure a precipitate than in the histon-mucoid preparation, but in other respects the results were practically identical.

4. The properties of this compound were then studied in exactly the same way as those of the histon-mucoid. The results of these tests established the following similarities between the two compounds. Both were insoluble in water.

Both gave perfect solutions in 0.05 per cent. Na_2CO_3 solution but incomplete solutions in 0.5 per cent. Na_2CO_2.

Both gave evidence of the formation of a soluble sodium-histon salt, in this case Na-histon-nucleoprotein.

Precipitation, solubility and color reaction tests were then conducted upon preparations of both histon-mucoid and histon-nucleoprotein. 0.05 per cent. Na_2CO_3 solutions of each were used where a soluble form was required. The results are given in the following tables. Results with other proteins are inserted for comparison.

After standing in the solvent for some time with frequent shakings the solutions were filtered off and tested with dilute NH_4OH and 0.2 per cent. HCl.

Solvents.	Histon.	Mucoid.	Yeast-nucleoprotein.	K-mucoid.	K-nucleoprotein.	Histon-mucoid.	Histon-nucleoprotein.	Albumin.	Globulin.	Alkali albuminate.	Proteose.	Peptone.	Coag. protein.
Distilled water	S	I	I	S	S	I	I	S	I	I	S	S	I
10 per cent. NaCl	I	I	I	S	S	I	I	S	S	I	S	S	I
Physiol. salt solution	I	I	I	S	S	I[1]	I[1]	S	?	?	S	S	I
Blood serum	I	I	I	S	S	?	?	S	I	I	S	S	I
0.05 per cent. Na_2CO_3	I	S	S	S	S	S	S	?	?	?	?	?	I
0.5 per cent. Na_2CO_3	I	S	S	S	S	I[2]	I[2]	S	S	S	S	S	I
0.2 per cent. HCl	S	I	I	I	I	I[3]	I[3]	S	S	S	S	S	I
Hot HNO_3	S	S	S	S	S	S	S	S	S	S	S	S	S
Cold HNO_3	I	I	I	S	S	I[4]	I[4]	S	S	S	S	S	S
5 per cent. Na_3PO_4	I	I	I	S	S	I	I	I	?	?	?	?	?
Conc. HCl	S	I	I	I	I	S	S	?	?	?	?	?	?

0.05 per cent. Na_2CO_3 solution of H-mucoid and H-nucleoprotein were used for tests.

Color reactions were applied to solid H-nucleoprotein and H-mucoid and to their 0.05 Na_2CO_3 solutions.

Reactions.	Histon.	Mcd.	N. Prot.	K-Mcd.	K-N.P.	H-Mcd.	H-N.P.	Albumin.	Globulin.	Alb'ates.	Proteoses.	Peptones.
Xanthoproteic	+	+	+	+	+	+	+	+	+	+	+	—
Biuret	+	+	+	+	+	+	+	+	+	+	Rose	+
Millon	—	+	+	+	+	—	+	+	+	+	±	—
Hopkins–Cole	—	+	+	+	+	—	—	+	+	+	±	—
Molisch	—	+	+	+	+	+	+	+	+	?	±	—
Liebermann	?	+	+	+	+	+	+	+	+	+	±	—

These tests were confirmed on new preparations.

[1] Physiol. salt. solution dissociates the compound; the filtrate gives a precipitate with 0.2% HCl.

[2] Na_2CO_3 (0.5%) dissociates the compound; the filtrate gives a precipitate with 2% HCl.

[3] 0.2% HCl dissociates the compound; the filtrate gives a precipitate with dil. NH_4OH.

[4] Cold conc. HNO_3 gives a white precipitate, probably the histon part.

Precipitant.	Histon.	K-mucoid.	K-nucleoprotein.	0.05% Na_2CO_3 sol. H-mucoid.	0.05% Na_2CO_3 sol. H-nucleoprotein.	Albumin.	Globulin.	Alkali albuminates.	Proteose.	Peptone.
Cold HNO_3.....	P	P	P	P	P	P	P	P	P	S
	S in excess									
Hot HNO_3.....	S	S	S	S	S	S	S	S	S	S
Picric acid.....	In alk P sol.	P	P	P	P	←—in acid sol.—→ P	P	P	P	S
KHgI.....	In alk P sol.	P	P	P	P	←—in acid sol.—→ P	P	P	P	S
Phospho molybdic acid	In alk P sol.	P	P	P	P	←—in acid sol.—→ P	P	P	P	S
Trichlor acetic acid....	In alk P sol.	P	P	P	P	←—in acid sol.—→ P	P	P	P	S
Blood serum.....	P	S	S	P	P	?	?	?	?	?
Sat. with NaCl.....	P	P	P	P	P	S	Partly	Partly	Partly	S
Sat. with Na_2SO_4.....	P	P	P	P	P	acid P	P	Partly	..	..
Sat. with $MgSO_4$.....	P	P	P	P	P	S	P	P	P	S
1/2 sat. $(NH_4)_2SO_4$....	P	P	P	P	P	acid S	P	?	?	S
Sat. with $(NH_4)_2SO_4$...	P	P	P	P	P	P	P	P	Some	S
Alcohol, 95 per cent...	S	S	S	S	S	P	P	P	P	S
Alcohol and salt.....	S	P	P	P	P	P	P	P	P	S
Alc. + ether + salt....	S	P	P	P	P	P	P	P	P	S
Dist. water.....	S	S	S	Partly	Partly	S	P	P	S	S
NH_4OH.....	P[1]	S	S	P	P	S	S	S	S	S
0.2 per cent. HCl.....	S	P	P	P	P	S	S	S	.	S

[1] Bang claims that histon is precipitated by NH_4OH only when salts are present. Our results tend to show

PREPARATION OF HISTON-OVOMUCOIDS.

In the following preparations the principal experiments were made with the ovomucoid obtained from hen's eggs as described on page 22. Parallel preparations were also run on the ovomucoid obtained from shad eggs. The results were somewhat variant but agreed in the main. The steps in the various preparations are self explanatory and are set down in detail in the following account:

Preparation of Histon-Ovomucoid. (hen-ovomucoid). *First Preparation.*—1. The ovomucoid used was material which had been dialyzed free from uncombined acid and salts, and was of such a degree of purity as to be practically soluble in alcohol which contained no salts. A little of this material was dissolved in distilled water forming a clear solution. Histon-HCl solution equally pure was added drop by drop. to the water solution of the ovomucoid. A precipitate formed at first, giving a turbid mixture which was allowed to stand for several days to permit the precipitate to settle out.

2. Three days later the mixture was still cloudy and no precipitate had deposited. This solution was then filtered and the filtrate tested. It failed to give a definite precipitate with ammonia but gave a heavy precipitate with phospho-tungstic acid. This result suggested a soluble compound but failed to account for the disappearance of the histon since, if it had been simply a mixture, the histon would have filtered through as well as the ovomucoid, being soluble in water. The result way have been due to the small amount of histon used in making the preparation.

3. The bulk of the filtered mixture was poured into 95 per cent. alcohol. No precipitate resulted in 48 hours. On stirring the mixture with a glass rod dipped in NaCl solution, a heavy precipitate settled out. This precipitate was filtered

off and the alcohol filtrate evaporated to dryness at 40° C. on a water bath.

4. A portion of the precipitate just obtained (3) was dissolved in water (solution took place readily) and this solution gave a good biuret test but no precipitate with ammonia. It was neutral in reaction. The bulk of the precipitate was then dissolved in water and acidified with HCl to see if the histon might be split off by the acid. The acidified solution gave no precipitate on adding ammonia to excess after standing a day. The bulk of the acidified solution was then poured into acidified alcohol. No precipitate resulted even after 3 drops of 10 per cent. NaCl solution were added. At the end of a week no precipitate had formed. At the end of that time 4 drops more of 10 per cent. NaCl were added. No precipitate formed in the next 24 hours. At the end of that time 10 drops more of the NaCl solution were added. By this time the alcohol had partly evaporated. To remedy this more alcohol was added. A precipitate at once separated out. This precipitate was filtered off and dissolved in water. The solution gave no precipitate with ammonia.

5. The alcohol filtrate from 3, when evaporated to dryness, left a residue which was insoluble in water and resembled the insoluble form of histon obtained by washing histon with alcohol and ether. Furthermore, when this substance was extracted with HCl, it gave a solution which was precipitated by ammonia. This precipitate was soluble in water. This result suggested gradual dissociation of the histon from the product and its conversion into the insoluble form.

While these results made it seem reasonable to suppose that the histon actually entered into combination with the ovomucoid the difficulty of purifying the compound and demonstrating the presence of the histon made the results extremely unsatisfactory. Consequently a second preparation was made.

Second Preparation.—1. The second preparation was made in the same way as the first but more care was used in adding the histon solution. As soon as an appreciable precipitate appeared, the mixture was allowed to stand. At the end of 24 hours a small amount of precipitate had settled out. This was filtered off, extracted with 0.05 per cent. Na_2CO_3 and the resultant solution filtered. The filtrate in turn gave a faint precipitate with ammonia.

2. The bulk of the mixture came through the paper in a turbid condition and the filtrate was divided into two portions. One portion was saturated with ammonium sulphate. The other was poured into 95 per cent. alcohol to which 3 drops of 10 per cent. NaCl solution were added. Heavy precipitates separated from both preparations on standing. Both precipitates were soluble in water and these water solutions gave heavy precipitates with ammonia.

These results seemed to indicate definitely that a soluble histon-ovomucoid compound was formed. The precipitate was apparently simply a portion that failed to go into solution under the saturated conditions. To make more certain of these facts the water solution of the ammonium sulphate precipitate was dissolved in water and put into a collodion bag with toluol and dialyzed against distilled water to remove all impurity. The water solution of the alcohol precipitate was poured into a large volume of alcohol to reprecipitate. It precipitated out only on the addition of a few drops of NaCl.

3. At the end of 24 hours the dialyzate from the ammonium sulfate precipitate was tested with ammonia. No precipitate resulted. The dialyzate was replaced with distilled water and was itself concentrated on a water bath at 40° C. The concentrated dialyzate gave no protein test. It gave a strong test for sulfate with $BaCl_2$ and was acid to litmus.

Twenty-four hours later the new dialyzate gave a strong

test for SO_4 but no precipitate with ammonia. The dialyzate was again renewed. After 48 hours the dialyzate was again tested. It gave a faint SO_4 test but no ammonia precipitate after concentrating.

The dialyzate was then replaced once more to remove all traces of sulphate. At the end of a week the solution in the bag was free from sulfate but it had precipitated and the precipitate was no longer soluble in water. When extracted with HCl it gave no ammonia precipitable solution. Hydrolytic (?) action had apparently entirely altered the character of the compound.

4. The portion reprecipitated by the alcohol was next filtered off. It again dissolved in water and the water solution in turn gave a heavy precipitate with ammonia. This result seemed to leave no doubt that histon formed with ovomucoid a soluble compound. A second portion of this alcohol precipitate was hydrolyzed with concentrated HCl, neutralized with solid KOH and tested with Fehling's solution for reduction. A slight precipitate formed. A second portion tested with Benedict's second solution gave a copious red precipitate. This latter result made certain that the compound contained ovomucoid as histon is not a glucoprotein.

Final Confirmation of the Production of a Histon-ovomucoid Compound.—1. A water solution of ovomucoid was mixed with a small amount of alcohol free of salt. No precipitate resulted.

2. An equal volume of histon-HCl was mixed with an equal volume of alcohol. No precipitate resulted.

3. The two alcohol mixtures were then poured together. A precipitate settled out, which when tested, was water soluble and gave an ammonia precipitate. Since the proportion of alcohol was not increased by this mixture and, furthermore, since equal portions of the alcohol solutions reserved

as controls failed to precipitate in this time, this result seemed to prove definitely that histon-ovomucoid was a substance different from both histon and ovomucoid in being more readily precipitable in alcohol than either of its components.

Preparation of Histon Ovomucoid (shad-ovomucoid). *First Preparation.*—Shad ovomucoid was used which was free of acid. This material dissolved readily in water and to it was added a solution of histon-HCl. The result was a dense white precipitate soluble in excess of histon solution. On standing this precipitate settled out as a white flocculent precipitate. On standing this precipitate became transparent and oily. The supernatant liquid was clear and gave no precipitate with ammonia though over 20 cc. of histon-HCl was used in the preparation. There seemed therefore no doubt that the histon had combined with the ovomucoid to form this precipitate.

2. The supernatant liquid was next decanted and water added. The oily precipitate on agitation by the water again settled out in flocculent form but on allowing this water mixture to stand the precipitate again assumed an oily condition. The supernatant liquid in this case gave no precipitate with ammonia but gave a good precipitate with phosphotungstic acid, indicating a separation of ovomucoid (?). The amount of the precipitate seemed to indicate a partial solution. When the portion of the supernatant liquid was decanted the portion next the precipitate gave a cloudiness with ammonia indicating a partial dissociation of histon or a solution of the product.

3. The precipitate was then washed into a bottle with water and toluol added. It was allowed to stand in the stoppered bottle for a week. At the end of that time complete solution of the precipitate had resulted. The solution was filtered off and tested for histon. It gave a precipitate with ammonia. This suggested one of two possibilities. Either the water

had dissociated the histon or the histon-ovomucoid had dissolved in the water.

4. The solution was precipitated with 95 per cent. alcohol with the addition of salt. The resulting precipitate was insoluble in water. It was washed with water until the washings have no biuret test. It was then allowed to stand in a stoppered bottle with toluol as before. This time the precipitate did not become oily in a week and also did not dissolve in the water.

At the end of that time the supernatant liquid gave no precipitate with ammonia. The precipitate was filtered off, extracted with HCl and this solution filtered off. It gave a precipitate with ammonia. These results seemed to indicate a compound of histon with the ovomucoid which while insoluble in water was capable of taking up water to form an oily state and eventually to dissolve in the water.

The second preparation confirmed the results with the first.

DIALYZABILITY OF HISTON.

Some of the results obtained suggested the possibility that histon was itself dialyzable. To settle this point the following experiments were carried out:

1. Celluloid bags were made by pouring collodion into an Erlenmeyer flask. After rinsing the flask with the collodion the excess was poured off, resulting in a thin film-like bag which may be separated from the inside of the flask with water and stirring rod. The bags thus formed were extremely rapid in their osmotic exchange and were used in these experiments.

2. One of these bags was filled with a neutral solution of histon-HCl. Distilled water was used as a dialyzate. Inside of an hour the water had risen in the bag 1/2 inch and the dialyzate gave a heavy precipitate with ammonia. In 24 hours the bag had taken up twice its volume of water. A

larger bag was then made and the solution transferred to that. 3. At the end of the next 24 hours the water had risen in the new bag and dialyzate contained a little histon. This porcess continued for some time with frequent renewals of the water. Histon continued to dialyze through for over a week. Toluol was used to preserve the material. At the end of this time the histon stopped coming through and the contents of the bag had precipitated and the precipitate was not readily soluble in water. It was also resistant to the action of HCl though an acid extract gave a faint ammonia precipitate. The supernatant solution gave a slight precipitate with ammonia. Evidently the histon had changed character and this accounted for the cessation of the dialysis.

The results in 2, and 3, were confirmed by using water solutions of purified histon and ammonium sulfate precipitates of histon dialyzed free of SO_4. These experiments are still in progress and enough has been done at present to make certain that histon is not only dialyzable but also to demonstrate that this dialyzability is not due to the presence of Cl or SO_4.

ANOTHER METHOD OF PURIFYING HISTON-MUCOID.

The results obtained in this laboratory from time to time indicated that purified moist mucoid products were practically soluble in 95 per cent. alcohol. In all cases as the purity increased precipitation became increasingly difficult and the salt had to be added to precipitate. Purified histon-HCl shows the same properties. This suggested a means of producing histon-mucoid on a large scale and a way of purifying the product.

Alcohol mixture of K-mucoid and histon-HCl were therefore made by mixing equal volumes of these two solutions separately with equal quantities of 95 per cent. alcohol. No precipitate results. When these two mixtures are brought

together care being taken to use equal amounts of each solution so as to not increase the proportion of alcohol for either solute, a copious precipitate of histon-mucoid at once separates out. This precipitate can then be filtered off and washed free from all excess of histon and mucoid. The result is a pure product of histon-mucoid. Experiments are now under way to still further purify this product by converting it into a water-soluble Na-histo-mucoid with 0.05 per cent. Na_2CO_2. The process will permit a more careful estimation of the properties of the compounds, elementary analysis of their structure, etc.

SUMMARY OF GENERAL CONCLUSIONS.

A. Alkaloidal-protein Compounds.

1. Strychnin and mucoid combine to form a compound whose protein and strychnin characteristics are demonstrable even after rigorous methods of purification are employed.

2. Mucoid combines with conin, piperidin, morphin, and anilin to produce water soluble and acid precipitable compounds. The amount of combined base in these compounds appears to be very small.

3. The continuous loss of strychnin from strychnin mucoid by dialysis, by ether extraction and by washing with chloroform seems explicable only on the basis of dissociation.

4. The results with caseinogen and yeast nucleoprotein indicate that they are capable of combining with a base in the same manner as mucoid.

5. Strychnin is especially suitable for observations in this connection because of the delicacy of the dichromate test for its detection.

B. Properties of Ovomucin (Hen).

1. Ovomucin is a glucoprotein mostly insoluble in water.

2. It dissolves somewhat in 10 per cent. NaCl, in 0.2 per cent. HCl and in weak alkali such as $Ca(OH)_2$.

3. Precipitation from acid or alkaline solution takes place at or near the neutral point.

C. Properties of Ovomucoid (Hen).

1. The water solution of this glucoprotein gives a good biuret test, froths strongly on shaking and does not thread nor become "thick flowing" on pouring.

2. Ovomucoid is precipitable by phosphotungstic and tannic acids. It is not precipitable by either 0.2 per cent HCl or 3 per cent. acetic acid.

3. The pure solution is distinctly acid to litmus.

4. *Shad* ovomucoid has the same properties.

D. Salts of Ovomucoids and Yeast Nucleoproteins.

1. Ovomucoid and yeast nucleoprotein form, with strychnin, compounds which are neutral to litmus, soluble in water and gave good protein tests.

2. These compounds easily give up their basic portions under such varying conditions as are supplied by dialysis and the presence of certain precipitants, indicating extreme lability and sensitiveness to environmental conditions.

3. The combination and dissociation of mucoid and nucleoprotein compounds take place under conditions which are similar to those realized in the living cell and it is quite within the realm of possibility that such reactions actually occur in living cells. The nature of such combinations is not so simple as that of a simple salt but bears a definite relation to it.

E. Properties of Histon.

1. Experiments confirm in the main, the findings of Bang and Huiskamp concerning the preparation and properties of histon.

2. Histon, contrary to Bang and confirmatory of Cohnheim, is precipitable from pure solution by ammonia but precipitation takes place very slowly and the presence of

a trace of salt hastens the precipitation to a remarkable degree.

3. Histon is dialyzable when distilled water is used as the dialyzate.

F. Protein Salts with a Histon Base.

1. Histon and tendomucoid combine to form a water *insoluble* compound which is soluble in 0.05 per cent. Na_2CO_3. This solution is apparently due to the formation of a soluble compound, sodium-histo-mucoid.

2. Histon and yeast nucleoprotein combine in a similar manner and the compound formed is similar in the above respects to histon-mucoid.

3. Ovomucoids enter into combination with histon to form compounds which are at least temporarily insoluble in water. These combinations in the presence of water and on dialysis, undergo changes which indicate great lability and powers of transformation.

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BIOGRAPHICAL.

Walter Hollis Eddy was born in Brattleboro, Vermont, August 26th, 1877. He was graduated from the Brattleboro High School in June, 1894, and in September of that year entered Amherst College. In June, 1898, he was graduated from that institution with the degree of Bachelor of Science. During 1898–1900 he held the position of science teacher in the Amherst High School and from October, 1899, to June, 1900, was acting principal of that school. In June, 1900, he resigned his position to accept the place of science teacher in the Passaic High School of Passaic, New Jersey. He remained there until September, 1903, when he was appointed assistant teacher of biology in the New York High School of Commerce. In February, 1904, he was made acting head of the department of biology in that school. In December, 1908, he was given the license of first assistant teacher of biology in New York City schools and in February, 1909, was made head of the department of biology in the High School of Commerce. This position he now holds.

In September, 1904, he matriculated as a candidate for the degrees of Master of Arts and Doctor of Philosophy in the Faculty of Pure Science, Columbia University. In June, 1908, he received the degree of Master of Arts from this University.

In July, 1908, he was appointed assistant in the Department of Biological Chemistry of the College of Physicians and Surgeons, Columbia University.

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